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(19) (CA) **CANADIAN PATENT** (12)

(54) Semen Sexing

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ABSTRACT:

In a method for sorting spermatozoa, spermatozoa are stained with Hoechst 33342. The fluorescence distribution of stained spermatozoa is complex: non-motile spermatozoa display a higher fluorescence than motile spermatozoa. The fluorescence profile of the motile spermatozoa is bimodal, and enables the spermatozoa to be sorted into distinct populations of motile spermatozoa.

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SEMEN SEXING

The present invention relates to a method of sorting living spermatozoa, and, for example, to a method of sorting living spermatozoa according to sex; that is, according to whether the spermatozoa bear an X or Y chromosome.

Throughout the following description, the lower case letters in parentheses refer to the following:

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- 15 Measurement of mammalian sperm deoxyribonucleic acid by flow cytometry. Problems and approaches. J. Histochem. Cytochem. 25, 763-773.
- 20 Flow microfluorometry is a convenient method for measuring the DNA content of mammalian cells (o). Spermatozoa, by virtue of their ease of collection from many species, their homogeneity and their haploidy, are particularly suitable
- 25 for such studies (p;e). To date, the majority of studies of the DNA content of spermatozoa have been carried out using fixed material stained with fluorochromes such as acridine orange, ethidium bromide, or mithramycin. Recently, the bisben-
- 30 zimidazole dyes Hoechst[®] 33258, Hoechst[®] 33342, and DAPI(4',6'-diamidino-2-phenylindole) have been introduced as quantitative fluorescent stains for DNA. These dyes, although they bind tightly to DNA, do not intercalate into the molecule and
- 35 hence are reputed not to disrupt its structure (k;l). These fluorochrome dyes are consequently capable of being used as quantitative vital stains

for DNA: Hoechst 33258 and Hoechst 33342 have been used as vital stains to distinguish phases of the cell cycle.

Since spermatozoa are tail bearing and motile they orientate with their long axis along the line of flow in a flow microfluorometry system (p). It has been concluded that an apparent bimodal DNA distribution in fixed acriflavine/Feulgen-stained bull sperm heads analyzed in such a system, is due to an orientation artefact (b), perhaps analogous to that previously described in (i) for the light scatter (size) artefact seen with chicken red blood cells (chicken RBC). Both of these artefacts can be by-passed or removed by the use of an appropriate nozzle which will control the orientation of flattened particles such as sperm heads or chicken RBC relative to the laser beam of the flow microfluorometry system (m ; b). As an alternative approach, distribution artefacts can be tested by sorting the population into its separate components and then reanalyzing them independently: if an artefact is involved, each reanalyzed peak will give a bimodal peak similar to that observed originally.

Various aspects of the invention are as follows:

A method of sorting living spermatozoa, the method comprising: the vital staining of spermatozoa, with a fluorochrome dye; subjecting the stained spermatozoa to a light source which causes fluorescence; and sorting the spermatozoa according to the fluorescence intensities associated therewith.

A method of sorting living spermatozoa, the method comprising: the vital staining of spermatozoa with a fluorochrome dye which binds tightly to DNA, does not intercalate the DNA molecule of chromosomes and hence does not disrupt the structure of DNA molecules; subjecting the spermatozoa to a light source which causes fluorescence and sorting the spermatozoa into different groups according to the fluorescence intensities associated therewith, one group mainly comprising X-chromosome bearing spermatozoa; and another group mainly comprising Y-chromosome bearing spermatozoa.

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The dye may be a bisbenzimidazole dye.

In an embodiment of the invention, the bisbenzimidazole dye Hoechst 33342 is used as a vital fluorescent stain for DNA which allows spermatozoa to remain motile after analysis. The fluorescence may be examined in detail using a commercially

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1 available fluorescence-activated cell sorter.

For a better understanding of the present invention, and to show how the same may be carried into effect, reference will now be made, by way of example, to
5 the accompanying drawings in which:

FIGURE 1 is a graph showing the distribution of fluorescence of bull spermatozoa stained with Hoechst 33342;

FIGURE 2 is a graph showing the distribution of
10 Figure 1, with a higher gain setting for the fluorescence-activated cell sorter;

FIGURE 3 is a graph showing the distribution of cockerel spermatozoa stained with Hoechst 33342 (5 µg/ml) in egg medium;

15 FIGURE 4 is a graph showing reanalysis of the peaks AI and AII in Figure 2;

FIGURES 5a to 5c are graphs showing the results of analysis with different orientations of the cells; and

20 FIGURE 6 is a table showing the effect of an orientating nozzle on FACS analysis of chicken RBC (size) and bull spermatozoa (fluorescence) compared to non-orientated cells.

In preparation for the analysis semen is collected, using an appropriate artificial vagina (c),
25 from Fresian and Hereford bulls. Shortly after ejaculation, semen is added to 1-2 volumes of egg or milk medium at 20-22°C. Milk medium is made according to the method described in (a), which comprises: centrifuging pasteurized milk at 2000 g for 10 min; removing the
30 cream; taking the underlying fat-free liquid from this slow speed spin; and pelleting the milk solids by centrifugation at 48000 g for 30 mins. The clear supernatant is then heated at 92-96°C for 10 min, and 0.125 g D-fructose/ml and antibiotics (10⁴ units
35 penicillin + 10 mg streptomycin sulphate per 100 ml) is added when the supernatant has cooled.

The spermatozoa are washed twice by centrifugation

X

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1 at 1000 g for 5 min followed by gentle resuspension of
the pellet in sufficient fresh medium to give a
concentration of, for example, 5×10^6 spermatozoa/ml.

5 Intact spermatozoa are then stained with Hoechst
33342 in milk medium at a concentration of 2 $\mu\text{g/ml}$ for
bull spermatozoa and 5 $\mu\text{g/ml}$ for cockerel spermatozoa, at
room temperature for 2-3 hours. The dye concentrations
may be determined empirically from subjective assessment
of optimal staining without overt cytotoxicity.

10 Flow microfluorometric analysis (g) is carried out
using a fluorescence Activated Cell Sorter (such as, for
example, FACS II: Becton Dickinson Electronics
Laboratories, Sunnyvale, California). The light source
for the FACS may be a 164-05 ultra violet-enhanced
15 argon-ion laser, (Spectraphysics), operated at 20 mW in
the u.v. range of wavelengths. Right-angle scatter of
u.v. laser light is prevented from entering the
fluorescence detector by a Wratten 2B filter. The FACS
is calibrated in the u.v. using glutaraldehyde-fixed
20 chicken red blood cells (f).

Samples of spermatozoa are analysed and sorted at
room temperature ($20-22^\circ\text{C}$) at a rate of up to 3500-5000
cells/sec, except during orientation experiments in which
the rate was reduced to <800 cells/sec. The sheath fluid
25 is Dulbecco's phosphate-buffered saline (pH 7.2;
containing Mg^{2+} and Ca^{2+}), but without stain.

The total fluorescence is calculated (in arbitrary
units), for example by a computer. Such a computer is an
LSI-11 based mini computer (Digital Equipment
30 Corporation, MA, USA) linked to the FACS, which
calculates the total fluorescence between channels 1 and
256 as follows (I):

$$35 \quad \text{Total fluorescence} = \sum_{1}^{256} \frac{\text{no. of cells in a channel} \times \text{channel no.}}{100} \quad (I)$$

Cells can be orientated in a single vertical plane

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1 at a predetermined angle to the laser beam by the method
described in (m). A (wedge shaped) sample injection
tube, with faces set at 20°C to the axis flow, has the
effect of making a (central) stream ribbon-shaped within
5 the sheath stream. Since the velocity of the sheath
stream is considerably higher than that of the sample
stream, the latter is drawn into a thin ribbon and the
flattened cells within this sample become orientated into
the plane of the ribbon.

10 Extrapolating from maximal flow rates which allow
successful orientation of chicken red blood cells, it has
been estimated, on the basis of cell (head) size and
viscosity of the medium, that successful orientation of
spermatozoa should occur providing that the flow rate
15 does not exceed 800 cells/sec, when using a sample
density of 5×10^6 /ml.

When necessary, heads may be removed from the
spermatozoa in milk medium by ultra-sonication for 5-10
min in a MSE ultrasonicator.

20 A population of bull spermatozoa stained for a
minimum of 2 hours with Hoechst 33342, (2 µg/ml Hoechst
33342) in milk medium shows a complex distribution of
fluorescence intensity, which is illustrated in Figure 1.
Data are given for spermatozoa in milk medium at ambient
25 temperature (20-23°C) for 2 hours and those killed by
being heated to 56°C for 5 min. There are two pairs of
peaks in the distribution, which have been labelled A and
B respectively. When examined microscopically, cells
from window B are non- (or only partly) motile, whereas
30 spermatozoa sorted from window A show active forward
motility. The likelihood that the B peaks represent dead
or moribund spermatozoa was tested by submitting a sample
of stained spermatozoa to 56°C for 5 min. This treatment
left the spermatozoa totally immotile and when the
35 fluorescence distribution of these immotile spermatozoa
was examined the entire distribution was concentrated in
the B peaks. A small peak seen between A and B in Figure

1 1 may represent spermatozoa in a transitory state between
A and B or the presence of a small percentage of diploid
spermatozoa (h).

5 Attention was concentrated on the A peaks of the
fluorescence distribution of stained bull spermatozoa by
running the FACS fluorescence gain at a higher setting
(Figure 2) so that the B peaks moved off-scale. The low
and high peaks of the observed bimodal fluorescence
distribution of the A peaks (AI and AIII) contained
10 approximately equal numbers of spermatozoa. The average
fluorescence of spermatozoa in peak AII was approxiamtely
30% higher than that in peak AI.

Qualitatively similar bimodal distributions are
also obtained using the same procedures as outlined above
15 for the bull, when analysing ejaculated rabbit, sheep,
goat and human spermatozoa.

When cockerel spermatozoa ($\sim 0.5 \times 4 \mu\text{m}$ heads, ~ 8
 μm tails) were stained with H33342 the resulting
fluorescence profile was quite different from that of
20 bull spermatozoa (Figure 3). The monophasic distribution
of fluorescence may reflect either the homogametic nature
of male birds or be due to the absence of an orientation
artefact in the cylindrically headed cockerel
spermatozoa. The bimodal fluorescence distribution of
25 bull spermatozoa may be due to a machine artefact,
analogous to that observed for light scatter (size)
analysis of chicken red blood cells, but reflect
underlying biological or physiological differences. An
investigation into the nature of the observed bimodality
30 was carried out by an analysis-sort-reanalysis of stained
spermatozoa and by the use of an "orientating" nozzle.

First, the living, Hoechst 33342-stained bull
spermatozoa with a fluorescence distribution similar to
that shown in Figure 2, were physically separated
35 (sorted) into AI and AII population. Each separated
population was then re-analysed and the respective
fluorescence distributions are shown in Figure 4.

1 Although the peaks were not clearly unimodal, the
spermatozoa from the AII fraction had a higher overall
fluorescence than those from AI as would be expected if
the spermatozoa in peak AI were from a population
5 different from that of those in peak AII. The low
fluorescent peak appearing at approximately channel 30
for both populations in Figure 4 was due to spermatozoa
from which the H33342 had leached. Fixation of
spermatozoa with buffered formal-saline (pH 7.4) before
10 or after staining or after they had been sorted failed to
reduce the leakage of dye. In 17 experiments in which
the spermatozoa in peaks AI and AII were separated, the
total fluorescence intensity of the reanalysed AII
population was $15.6 \pm 2.9\%$ greater than that of the AI
15 population. For a comparison, the same experiment was
performed using chicken RBC. It is known that the
apparent bimodal size distribution of the chicken RBC is
an artefact related to the orientation of individual
cells to the laser beam. When the chicken RBC were
20 sorted into two peaks on the basis of scatter, each
separated peak gave the same bimodal distribution as the
original, unsorted, material when reanalysed.

Second, an orientation nozzle similar to that
described in (m) was used to analyse bull spermatozoa.
25 The efficiency of the nozzle was tested using a
light-scatter analysis of chicken RBC (1200 cells/sec).
Figure 5 shows results using an orientating nozzle for
(a) chicken RBC and (b, c) bull spermatozoa. In Figure
5a) peak 1 was obtained when the sample ribbon was
30 parallel to the laser beam; peak 2 was obtained when the
sample ribbon was at right angles to the laser beam; and
peak 3 for randomly orientated cells. In Figure 5b) peak
1 was obtained when the heads of the spermatozoa were
orientated edge on with respect to the laser beam and
35 peak 2 when the sample was rotated through 90° in the
axis of the flow (laser beam intersecting the broad side
of head); randomly orientated cells are indicated by 3.

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1 In Figure 5c) the bimodal distribution of fluorescence
intensity of intact Hoechst 33342-stained bull
spermatozoa was not affected by altering the orientation
of the sample ribbon: the distributions of randomly
5 orientated cells overlapped. The scatter distribution of
chicken RBC (Figure 5a) was affected by orientating the
cells with their edges parallel to or at right angles to
the laser beam. A similar effect was observed when sperm
heads were passed through the orientating nozzle and the
10 effect on the fluorescence profile examined. Although
bull spermatozoa have flattened heads, they did not
display a biphasic scatter (size) profile similar to that
seen when analysing chicken RBC. Nevertheless, the heads
of bull spermatozoa could be positively orientated, since
15 the resulting fluorescence profiles were monophasic and
did not overlap; (Figure 5b). In contrast, the bimodal
fluorescence distribution of intact bull spermatozoa
stained with Hoechst 33342 was not altered by rotation of
the nozzle (Figure 5c). The percentage of cells within
20 each peak is shown in Figure 6.

Bull spermatozoa stained with Hoechst 33342 in
milk or egg medium show a complex profile of
fluorescence when analysed on the FACS. The observed
fluorescence distribution of particles the size of
25 spermatozoa ($\sim 2 \times 5 \times 10 \mu\text{m}$ head, $40 \mu\text{m}$ tail) can be
divided into three main areas: (1) unstained material,
(2) a pair of highly fluorescent peaks (B) shown to
consist of dead or moribund spermatozoa, and (3) a pair
of peaks (AI and AII) with intermediate fluorescence
30 which consist of spermatozoa with normal forward
motility. Attention has been concentrated on peaks AI
and AII.

An increased staining of non-viable cells by
Hoechst 33342 similar to that seen here for bovine
35 spermatozoa has previously been reported for dead or
dying lymphocytes stained with the same dye. It has been
suggested (n) that the increased uptake of stain was due

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1 to a breakdown of the integrity of the cell membrane at
cell death. This may be the mechanism responsible for
the observed increase in fluorescence of dead spermatozoa
although it is possible that the normally tightly packed
5 DNA in the nucleus becomes disorganized and this
contributes to the increased staining. However,
preliminary fluorometric studies suggest that a
considerable increase in the fluorescence intensity of
Hoechst 33342 occurs as the pH decreases, irrespective of
10 whether the dye is bound to DNA, protein or is free in
solution. This observation suggests that the B peaks may
arise because of increased nuclear acidity at death.

The bimodal distribution observed in the Hoechst
33342 staining of viable spermatozoa (peaks A) is
15 probably a consequence of the biologically different
kinds of spermatozoa in the normal ejaculate.
Accordingly a comparison of the fluorescence profiles of
mammalian and bird spermatozoa, which are heterogametic
and homogametic respectively shows the cockerel
20 spermatozoa to have a unimodal distribution; Figure 5
illustrates that although the heads of spermatozoa can be
orientated, the bimodal fluorescence distribution of
Hoechst 33342-stained intact live spermatozoa is
apparently independent of the orientation of the sperm
25 heads around their long axis; and peaks AI and AII
(Figure 4), although not clearly unimodal, are of
predictable fluorescence in that spermatozoa separated
from peak AII fluoresce more brightly than those from AI:
a difference which averages at about 15%. If bimodality
30 had a machine orientation artefact the separated
population would be expected to have identical (bimodal)
distributions.

Thus the observed bimodality of fluorescence
distribution indicates the presence of two
35 physiologically or biologically different sub-populations
of viable spermatozoa. The sub-populations (AI and AII)
may reflect spermatozoa at distinct stages of late

1 maturation or the difference between X- and Y- chromosome
bearing spermatozoa. Experimental work with rabbits has
yielded a 3.5:1 ratio of correct sex to incorrect sex,
5 which is very close to the ratio which would be predicted
from a theoretical estimate of the overlaps between the
two sorted peaks. The above described method thus has a
useful application in sorting spermatozoa according to
whether they are X- or Y- chromosome bearing spermatozoa.

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The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

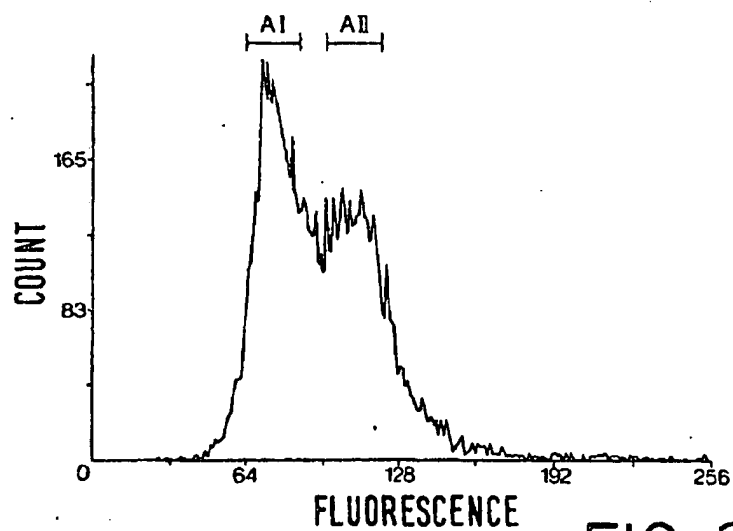
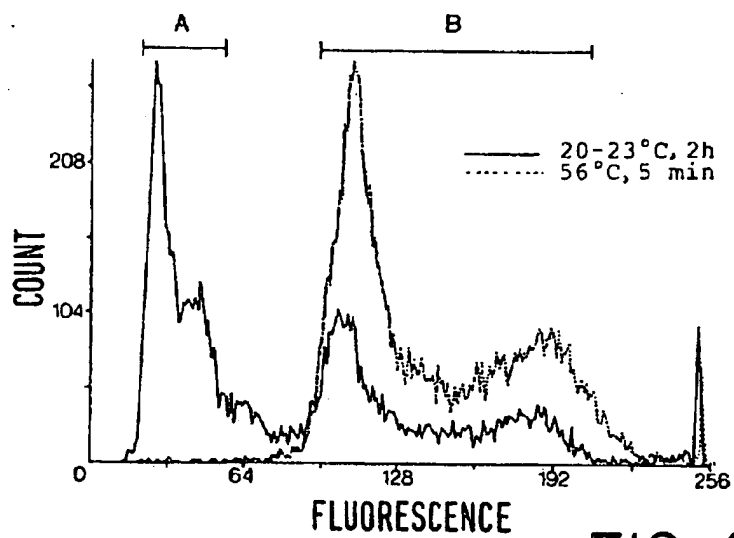
1. A method of sorting living spermatozoa, the method comprising: the vital staining of spermatozoa, with a fluorochrome dye; subjecting the stained spermatozoa to a light source which causes fluorescence; and sorting the spermatozoa according to the fluorescence intensities associated therewith.
2. A method according to claim 1, wherein the dye is a bisbenzimidazole dye.
3. A method according to claim 1 or 2, wherein the spermatozoa are of one of the following mammalian genera or families; bovidae; equidae; capridae; ovidae; lagomorphidae; and hominidae.
4. A method according to claim 1 or 2, when used to separate spermatozoa into different groups; one group mainly comprising X-chromosome bearing spermatozoa; and another group mainly comprising Y-chromosome bearing spermatozoa.
5. A method as claimed in claim 1, wherein the spermatozoa are sorted by a flow microfluometric process.
6. A method of sorting living spermatozoa, the method comprising: the vital staining of spermatozoa with a fluorochrome dye which binds tightly to DNA, does not intercalate the DNA molecule of chromosomes and hence does not disrupt the structure of DNA molecules; subjecting the spermatozoa to a light source which causes fluorescence and sorting the spermatozoa into different groups according to the fluorescence intensities associated therewith, one group mainly comprising X-chromosome bearing spermatozoa; and another group mainly comprising Y-chromosome bearing spermatozoa.
7. A method according to claim 6, wherein the dye is a bisbenzimidazole dye.

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8. A method according to claim 5 or 6, wherein the spermatozoa are one of the following mammalian genera or families; bovidae; equidae; capridae; ovidae; lagomorphidae; and hominidae.

9. A method as claimed in claim 6, wherein the spermatozoa are sorted by a flow microfluorometric process.





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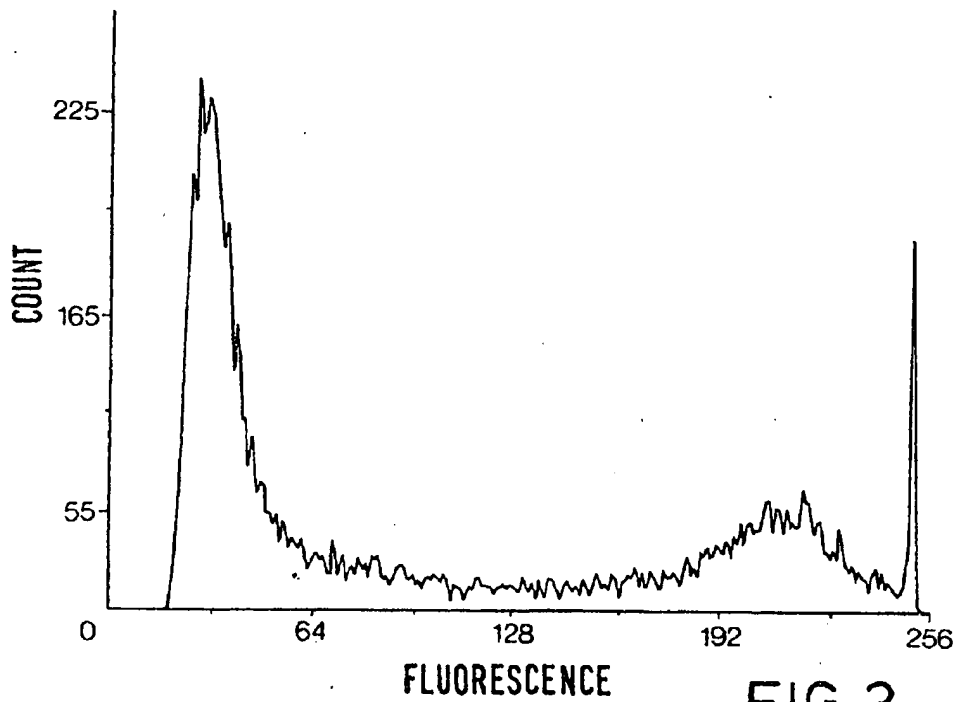


FIG. 3

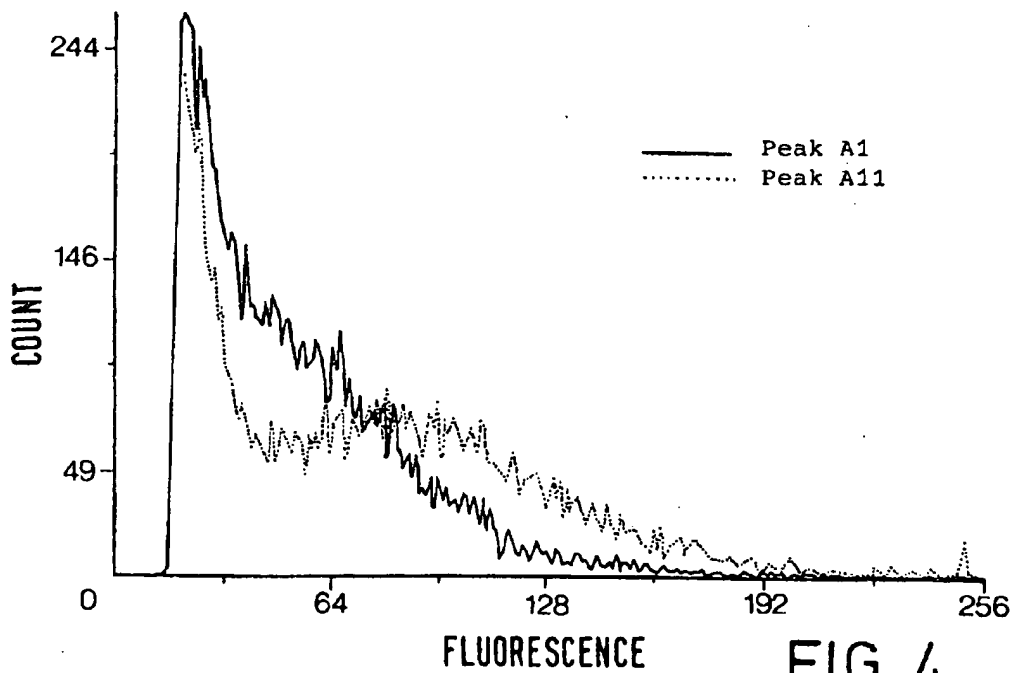


FIG. 4

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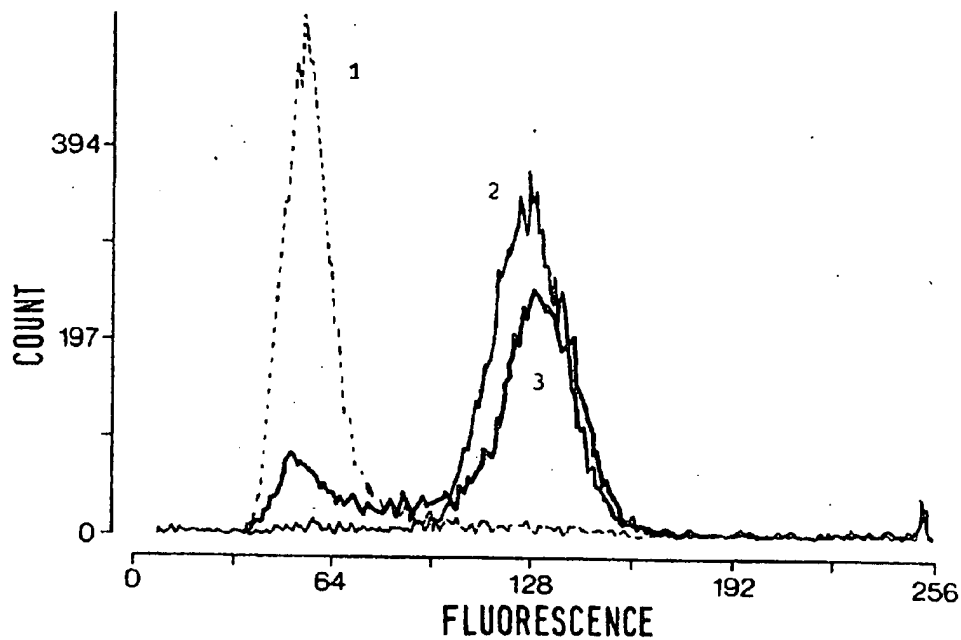


FIG. 5a

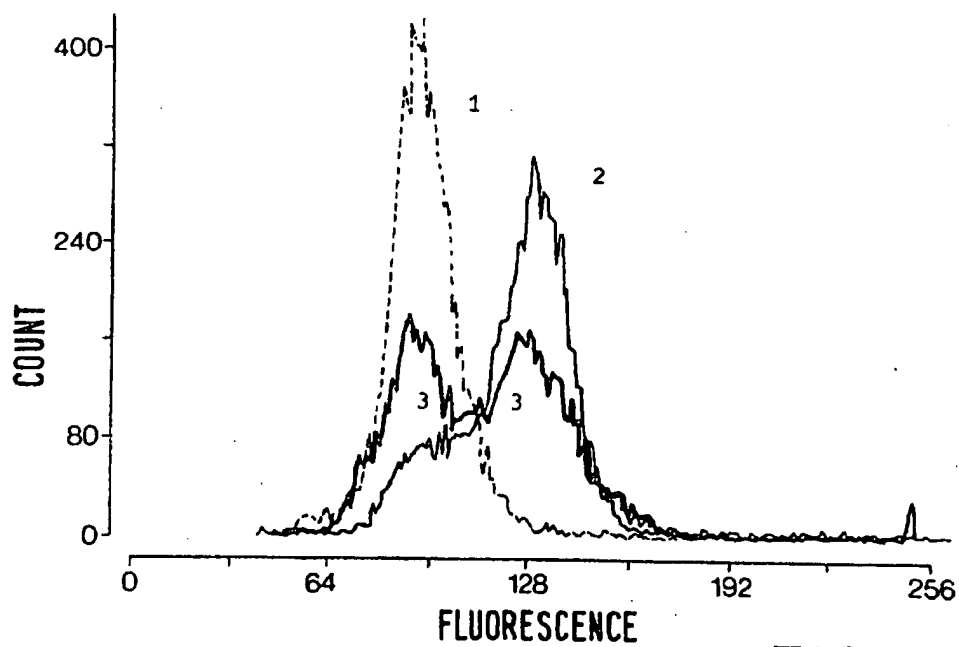


FIG. 5b

Sim: J. Bunnell

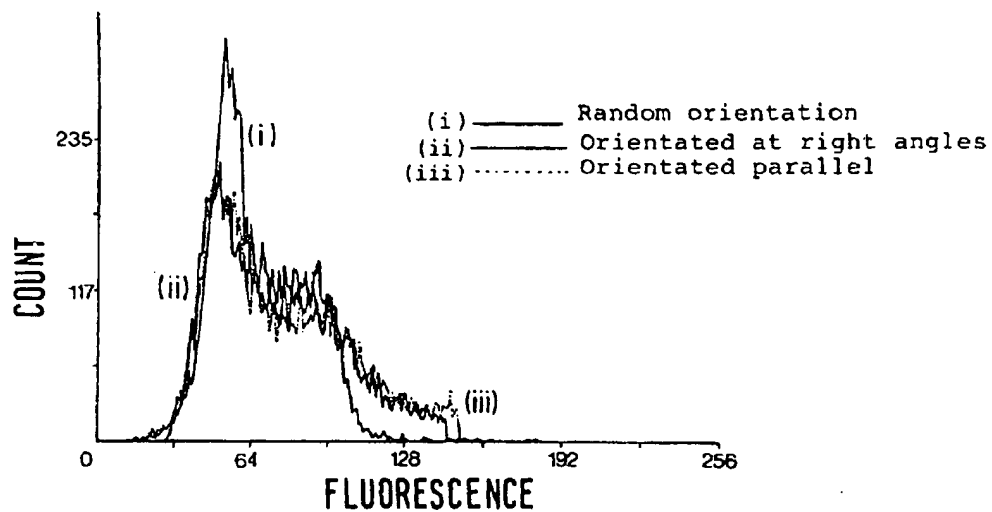


FIG. 5c

Orientation of cell to laser beam	Chicken RBC		Bull spermatozoa			
			Heads		Intact	
	Low Peak	High Peak	Low Peak	High Peak	Low Peak	High Peak
Random (normal nozzle)	22	78	43	57	51	49
Narrow side	94	6	90	10	59	41
Broad side	3	97	22	78	51	49

The values are the no. of cells in each peak of the distribution expressed as a % of the total.

FIG. 6

Sim: 4. G. G. G.